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PRINCIPAL INVESTIGATOR: Kevin Taylor

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, Ohio 45221-0061

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Cincinnati, Ohio 45267-0529

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Transforming Growth Factor a (TGFα) is an oncogenic growth factor which drives estrogen-independent breast tumor proliferation via an autocrine loop. Its receptor, epidermal growth factor receptor (EGFR), is also a proto-oncogene, which in breast tumors may be overexpressed by gene amplification. In contrast, in normal breast TGFa and EGFR are expressed in an estrogen-dependent, cell-specific manner as part of an intercellular paracrine loop. The function of this paracrine loop is unknown. This proposal defines the role of TGFα/EGFR in the early steps of tumor progression prior to invasion by defining the cell-specific expression of TGFα and EGFR in normal and premalignant breast tissue. We have shown TGFα mRNA expression in eight breast cancer cell lines by either Northern blot analysis or in situ hybridization. In addition, we have shown TGFα protein expression by Western blot analysis in the eight cell lines and in freshly isolated tissue. Interestingly, fresh tissue samples express predominantly the soluble (6 kD) form of TGFα, unlike the cell lines which express mostly the transmembrane form of TGFα. Although more tissue samples are needed, this may indicate a difference in TGFα processing between transformed and non-transformed breast epithelium.

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INTRODUCTION

Breast Cancer As a Process. The etiology of breast cancer can be defined in terms of the genetic changes which predispose breast epithelium to disregulated growth. These genetic changes may be inherited or the result of environmental insults. Regardless of the mechanism, these genetic changes result in the increased expression of oncogenes and the loss of expression of tumor suppressor genes, leading to uncontrolled proliferation (1-3). Endogenous estrogens are among the environmental influences which contribute to breast tumor development. Estrogens play a significant role in the normal growth and development of the breast (4). However, there is now little doubt that estrogenic stimulation of normal breast epithelial proliferation is central to the etiology of breast cancer development (5). Without estrogen, women do not get breast cancer. The mechanism by which estrogen-induced proliferation predisposes to specific genetic changes is not known. Furthermore, the mechanism of estrogen interaction with proto-oncogene products in normal breast tissue has not been extensively examined. This proposal focuses on the interaction of estrogen and one such proto-oncogene, transforming growth factor α (TGFα), during tumor development.

TGFα in Breast Tumorigenesis. Transforming Growth Factor α is commonly expressed at high levels in invasive breast cancer (6-8). This protein is a dominant oncogene, i.e. when overexpressed in breast epithelium, it produces a fully transformed cell (7-11). However, TGFα is also expressed at high levels in normal breast epithelium, and its synthesis is driven by estrogen (12-17). The function of TGFα in normal epithelium, therefore, appears to be quite different than that in tumor cells. Explaining this dichotomy is one goal of this proposal.

Many oncogenes encode growth factors, therefore the effects of growth factors in malignant cells have been well characterized (18). For instance, we know that the effects of growth factors (including TGFα) are dependent on the differentiated state of the cell, that is a growth factor which causes proliferation at one state of cellular differentiation may induce quiescence in a cell in another state (19). TGFα is expressed in normal epithelium, during pre-malignant proliferation, and in both in situ and invasive carcinoma (8, 20-21). Yet its function is clearly different over this progression to tumor formation. Understanding the role TGFα plays in normal and premalignant breast tissue is critical to understanding the mechanism through which TGFα mediates its effects in tumors.

Breast Histology. To understand the function of TGFα in the breast, one must understand normal breast histology. Breast

epithelium is composed of several epithelial cell types (22). Ducts are lined by luminal cells and myoepithelial cells. The luminal cells of the duct are thought to be involved in carcinogenesis (23). However, myoepithelial cells are occasionally transformed, also. This is not entirely surprising since myoepithelial and luminal cells are derived from the same stem cell population (24-25). For the most part, myoepithelial cells are not found in invasive carcinoma and when ducts are filled with carcinoma prior to invasion, at a stage called "in situ carcinoma," the myoepithelial cells are lost, or at best, greatly reduced in number (23). The milk-forming unit, or the lobule of the breast, is also composed of two epithelial cell types, alveolar cells and myoepithelial cells (22). The alveolar cells are the source of lobular carcinoma, a disease much less common than invasive ductal carcinoma (26). The lobular myoepithelial cells are morphologically similar to the ductal myoepithelial cells; however, biochemically they display some differences (our observations). Hormone-sensitive connective tissue composed of fibroblasts and myofibroblasts surrounds the epithelial cells of the breast. The role these cells play in the process of carcinogenesis is completely unknown. Each of these cell types expresses proto-oncogenes during the normal development and maturation of the breast. The regulation of this expression is the process which goes awry in tumorigenesis. To understand this regulation, we must examine the cell-specific expression of these proto-oncogenes and define the characteristics of normal breast which participate in this regulation.

Our preliminary data suggests that TGFα and its receptor (epidermal growth factor receptor or EGFR) function by two different mechanisms in normal breast. Here we show that the luminal cells of normal, quiescent breast epithelium express TGFα, but not EGFR; therefore, unlike tumor cells, the luminal cells can not respond to secreted TGFα in an autocrine fashion (Figure 1). Myoepithelial cells and adjacent stromal cells express EGFR normally; therefore, in normal breast the target for TGFα production is the myoepithelium and connective tissue. The cellular responses to this stimulation by TGFα are not known. Between quiescent epithelium and invasive tumor lie intermediate states of proliferation/transformation. Proliferative breast diseases of various morphologic types carries an increased risk of carcinoma and is considered a "premalignant" phenotype (27). In the literature and in our own experience, luminal EGFR expression is associated with some proliferative and cystic breast diseases (28). In particular, luminal cells with EGFR also express the estrogen receptor (ER) (28). Thus, alterations in EGFR synthesis may appear very early in proliferating breast, driven by estrogen. In fact, antisense studies with TGFα suggest

that induction of TGFα synthesis may be the main mechanism for estrogen-induced proliferation in this cell type (15). By the time breast epithelium has the characteristics of in situ carcinoma, TGFα and EGFR clearly co-localize in the luminal cells. The luminal cells up regulate EGFR and in the fully transformed state, respond to TGFα in an autocrine loop. At this stage there is an inverse correlation between EGFR and ER expression, consistent with the concept that over-stimulation of the TGFα/EGFR pathway leads to estrogen-independent growth (29). Some have suggested that cell transformation by EGFR is the result of a "threshold" level of EGFR expression (10). In this case, one would expect quantitatively increased expression in in situ carcinoma or invasive disease relative to these "premalignant" lesions. While the luminal cell is being driven to proliferate, the myoepithelial cell is lost during the transition from premalignant proliferative growth to in situ carcinoma. The mechanism by which these cells are lost, and the consequences of their loss are not known.

Here, we propose that TGFα effects on cells with luminal differentiation are proliferative; whereas, the effects on adjacent myoepithelium and connective tissue are to maintain cellular differentiation. The results of other investigations support this concept. First, MMTV-regulated TGFα expression in transgenic mice leads to uncontrolled luminal cell proliferation, with a normal relationship to myoepithelium. Cystic hyperplasias were particularly common in these animals, and development of adenocarcinoma was stochastic with time, consistent with TGFα functioning as a tumor promoter (30). Second, TGFα function is different depending on whether it acts via a paracrine or an autocrine mechanism. For instance, transfection with transmembrane TGFα leads to transformation whereas, exogenous TGFα, does not (11). Third, other extracellular molecules with TGFα-like domains are known to regulate differentiation through EGFR in many animals, including invertebrates (31). With myoepithelial differentiation, we believe the cells are no longer able to respond to TGFα by proliferation, and in fact that the function of TGFα on myoepithelium is to maintain cellular quiescence/differentiation. Conversely, myoepithelium is known to induce differentiation in breast cancer cell lines (32). Therefore, the presence of myoepithelium in breast tissue may be important in regulating luminal cell proliferation/differentiation. We propose that myoepithelial loss in tumor development results in loss of growth inhibitory signals which may or may not be related to TGFα/EGFR.

In this proposal we will explore the role of TGFα in normal and proliferative breast tissue. Understanding the normal role of TGFα in tumor production is critical for the following reasons. First, TGFα is a major estrogen-responsive gene (33); therefore, to understand estrogen-promotion of tumorigenesis, one must know the consequence of estrogen-induced gene expression. Second, TGFα function changes during the process of tumorigenesis. In normal tissue TGFα operates in a paracrine fashion and may be critical for maintaining normal lobular growth. In fact, there is now evidence that lactation is regulated by TGFα (34). However, in tumors TGFα leads to estrogen-independent growth (9). The effects of TGFα in the steps in between, from normal cell growth to invasive disease, have not been studied. Finally, current experimental therapies target various points in the TGFα signal transduction pathway. For instance, TGFα-EGFR stimulates cells through the MAP kinase pathway (35). This signal transduction pathway involves the proto-oncogene, ras, as well as two kinases which have been well characterized, protein kinase C (PKC) and protein kinase A (PKA) (36). The action of PKC is of particular note, since when EGFR is activated, it results in PKC activation (37-39). As part of an inhibitory feedback loop, PKC then phosphorylates and inhibits the action of EGFR, preventing over stimulation of the cell (40). These are exactly the types of pathways which one would predict are destroyed during the progression to malignancy. Current clinical trials include the use of Bryostatin, an agent which inhibits PKC (41). Given that TGFα can induce proliferation or differentiation, based on the differentiation state of the receptor cell, it is likely that the signaling pathways downstream of EGFR change during tumorigenesis. Therefore, it is critical that we understand the role TGFα is playing in specific breast cells at the time therapies affecting these pathways are instituted.

In this proposal, we build on our previous work, examining oncogene expression during tumor development and metastasis (Figure 1). Here we shall define the cell-specific expression of TGFα and EGFR in histologically similar premalignant states from women who do or do not progress to invasive tumor. Expression will be correlated with histopathologic types of proliferative breast disease, extent of disease types appearing concurrently, and estrogen receptor status. The observation that TGFα and EGFR must form a paracrine loop between myoepithelial and luminal cells is the result of careful evaluation of benign breast tissues for oncogene expression during a study comparing oncogene expression in benign, invasive, and metastatic tumors (43). In addition, the PI's mentor has collected an extensive set of premalignant breast tissue which are used for these studies. The results of this study will provide an invaluable database of

cell-specific gene expression in vivo which can then be correlated with in vitro tissue culture work.

RESULTS

1. Prepare riboprobes for in situ hybridization: subcloning of cDNAs, sequencing constructs. (Month 0-6).

The TGFα riboprobe was constructed by first subcloning 925 base pairs of TGFα cDNA (Genbank 190170) into the PGEM 3Z plasmid vector (Promega). Once cloned into PGEM vector, orientation and fidelity of cloning were verified by sequence analysis (Department of Molecular Genetics, Core Facility, University of Cincinnati College of Medicine, Cincinnati, Ohio). Sequence analysis showed the TGFα construct is oriented in the 5′ to 3′ direction relative to the T7 promoter in the vector. Therefore, transcription from the T7 promoter transcribes the antisense probe and SP6 transcribes the sense probe.

Conclusion: 925 base pairs of the TGFα gene has been sub-cloned into the PGEM 3Z plasmid and is in the 5′ to 3′ orientation relative to the T7 promoter.

2. Identify TGFα splice variants in breast cancer cell lines: Northern/RNase protection. (Month 6-9).

Initially, the riboprobe was labeled using the Genius Nonradioactive Nucleic Acid Labeling and Detection System (Boehringer-Mannheim Corp., Indianapolis, IN). The antisense TGFα digoxigenin labeled riboprobe was able to detect both the 4.8 kb and 1.6 kb mRNA transcripts on Northern blots of A431, and Hs-578-Bst, MDA-MB-453 cell lines (ATCC, Rockville, MD)(Figure 2). Due to the poor sensitivity of the digoxigenin labeled riboprobe(discussed in more detail under in situ hybridization section), labeling was switched from a nonradioactive to a radioactive method. The TGFα riboprobe is now labeled with ^{32}P-CTP according to a standard Promega protocol. To date, 25 RNA filters have been prepared and include the following eight breast cell lines: Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468,and MCF-10A. These filters will be probed with the ^{32}P-UTP labeled TGFα riboprobe.

Conclusion: RNA filters on eight cell lines were prepared, and three of which have been probed with the digoxigenin labeled riboprobe. The three cell lines (A431, Hs-578-Bst,and MDA-MB-453) express the expected sized transcripts as quoted in the literature (44). Due to the insufficient sensitivity of the digoxigenin labeled probe a new ^{32}P-CTP labeled riboprobe was made and will be used to probe RNA filters on all eight cell lines.

3. Identify TGFα protein sizes in breast cell lines: Western blot. (Month 6-9).

Protein was isolated from the same eight cell lines from which RNA was isolated (see above) and Western blots performed. The blots were probed with an anti-TGFα antibody (213-4.4, Oncogene Research Products, Cambridge, MA), which detected both the transmembrane (approximately 21 kD)and the soluble (approximately 6 kD) forms as expected (Figure 3)(44). The primary form detected in these cell lines was the transmembrane bound form. Conclusion: Protein was isolated from Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468,and MCF-10A cell lines, and all predominantly express the transmembrane form.

4. Repeat 2 and 3 in normal breast cells or organoids (Northern and Western blots). (Month 9-12).

As mentioned previously, 25 RNA filters have been prepared. These filters include RNA from eight cell lines and six fresh tissue samples. In addition to the Westerns run on the eight cell lines, Westerns were also performed on cultured fibroblasts, and six fresh breast tissue specimens (Figure 3). Interestingly, unlike the cell lines or cultured fibroblasts, freshly isolated tissue expressed mostly the soluble (6 kD) form of TGFα. We are now doing experiments to see if this difference in TGFα protein expression is due to culturing of the cells (i.e. a culture artefact), or if an underlying difference exists between transformed versus non-transformed epithelial cells. Conclusion: Both RNA and protein were isolated from six fresh tissue samples. RNA filters have been prepared on tissue samples and are waiting to be probed. Western blots were performed on protein isolated from both cultured fibroblasts and fresh tissue specimens. The fresh tissue specimens expressed mostly the soluble form of TGFα, while cultured fibroblasts expressed mostly the transmembrane form.

5. Develop splice-specific probes: subcloning/RT-PCR. (Month 9-12).

Due to the poor sensitivity of the digoxigenin labeled riboprobes we are repeating our Northern blots using ^{32}P labeled probes. To date, no potential splice variants have been identified.

6. Examine normal and proliferative breast tissue for cell-specific expression of TGFα: in situ hybridization and RT-PCR. (Month 12-24).

Paraffin embedded tissue from 90 patients have been collected and characterized. TGFα and EGFR immunohistochemistry have been performed on all samples. These data are now being analyzed. A digoxigenin-labeled riboprobe was prepared for in situ hybridization and showed good hybridization using paraffin embedded cell blocks of TGFα producing A431 cells (Figure 4). However, based on our observations and those of others the low level incorporation of digoxigenin-labeled UTP made the sensitivity of the probe insufficient to be used for in situ hybridization on paraffin embedded tissues. Serial dilution experiments indicated we were only able to consistently identify 100 or more picograms of riboprobe (data not shown). The literature indicates a detection level of 0.1 picograms for in situ hybridization is required(44). Thus, to improve the sensitivity of our riboprobe we are now labeling with ^{35}S-UTP. Conclusion: In situ Hybridization of digoxigenin labeled probe detected TGFα mRNA in A431 cells, but is not sensitive enough to perform ISH on paraffin embedded tissues. Work with radioactive probes is in progress.

7, 8, & 9.
These are goals for future years and are not addressed at this time.

DISCUSSION

We have subcloned 925 basepairs of the TGFα gene into the PGEM 3Z plasmid and have made digoxigenin-labeled riboprobes. The antisense probe specifically identified TGFα mRNA in paraffin embedded A431 cells. However, due to the insufficient sensitivity, riboprobes are now labeled with either ^{35}S-UTP (in situ hybridization) or ^{32}P (Northern blot). Twenty-five RNA filters have been prepared from eight cell lines (Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468,and MCF-10A)and six fresh breast tissue specimens. Northern blots probed with the digoxigenin labeled TGFα riboprobe identified both the 4.8 kb and 1.6 kb TGFα mRNA in three cell lines (A431, Hs-578 Bst, & MDA-MB 453).

Protein was isolated from the same eight cell lines from which RNA was isolated (see above) and Westerns performed. Westerns were also performed on freshly isolated tissue and cultured breast fibroblasts. To date, there is Western data on eight cell lines, 1 sample of fibroblasts, and five samples of fresh tissue. The cell lines and the fibroblasts expressed mostly the transmembrane form of TGFα, while the fresh tissue expressed mostly the soluble form. This difference in TGFα protein expression may be due to adaptive mechanisms from long term in

vitro culture conditions, or may reflect difference in transformed versus non-transformed epithelial cells. In either circumstance, more data need to be obtained, including testing conditioned media from cultured cell lines and comparing matched samples of freshly isolated organoids and after short term culture.

Lastly, we have histologically characterized 90 paraffin embedded breast tissue samples. TGFα and EGFR IHC has been performed on all samples and the data are now being analyzed. These data should provide meaningful information TGFα and EGFR expression during tumorigenesis in breast epithelium.

MATERIALS & METHODS

Tumor cell culture: Breast tumor cell lines Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468,and MCF-10A were obtained from ATCC and cultured according to their protocols. Cells are passaged weekly using trypsin-EDTA.

Organoid isolation: Breast epithelium (organoids) is dissected from both mastectomy and reduction mammoplasty specimens, minced, and enzymatically digested with collagenase as previously described (45). Briefly, tissue is obtained within 1 hour of surgery, minced with opposing scalpels to 1 mm3 pieces, and enzymatically digested for 12 to 18 hours at 37 oC in a rotary shaker (60 rpm) with 150 U/ml Collagenase Type I (Gibco), 100 U/ml Hyaluronidase (Gibco) in CDM3 media. CDM3 media consists of DMEM/f12 (1:1), 2.6 ng/ml selenium, 100 ng/ml EGF, 0.1 μg/ml fibronectin (all from Collaborative Research, Bedford, MA), 3 μg/ml insulin, 25 μg/ml transferrin, 10^{-10} M estradiol, 10^{-6} M hydrocortisone, 10^{-8} M cAMP, 10^{-8} M triiodothyronine, 10^{-4} M ethanolamine, 10^{-4} M phosphoethanolamine, 0.1% BSA, 10 μg/ml ascorbic acid, 20 μg/ml fetuin (all from Sigma, St. Loius, MO), and 1X trace element mix (Biofluids, Rockville, MD). Following digestion, epithelial organoids and fibroblasts are separated from vascular fragments and from one another by sedimentation.

Non-radioactive labeling of riboprobe: cDNA in the PGEM 3Z plasmid is linearized by restriction enzymes: BAMH1 for the sense probe yielding a 767 basepair fragment, and APA1 for the antisense probe yielding a 734 basepair fragment. 1 ug of linearized template was added to 10mM ATP, 10 mM GTP, 10 mM CTP, 6.5 mM UTP, and 3.5 mM digoxigenin labeled UTP, 20 Units of T7 and Sp6 Polymerase each in 40 mM Tris-HCl pH 8.0, 6 mM MgCl2, 10 mM dithioerythritol (DTE), 2 mM spermidine, 10 mM NaCl, and 0.1 U/ml RNase inhibitor. The reaction mixture is incubated for 12 hours at 37 °C and the reaction is terminated by adding RQ1 RNase-free DNase for 15 minutes at 37 °C. The riboprobe is isolated by phenol-chloroform extraction and ethanol precipitation.

Estimating yield of digoxigenin labeled nucleic acids: The riboprobe is serially diluted and spotted onto a Magna Graph Nylon Transfer Membrane (MSI Inc., Westboro, MA). Once dry the nucleic acids are cross-linked to the membrane by a brief exposure to UV. The Genius 3 Nucleic Acid Detection Kit (Boehringer-Mannheim) is used to identify the riboprobe. Briefly, the membrane is soaked in Blocking Reagent (Boehrninger-Mannheim) for 5 minutes, incubated for 10 minutes with the anti-digoxigenin alkaline phosphatase antibody diluted 1:5,000 in Blocking Reagent, washed two times with washing buffer (10 mM Maleic acid; 15 mM NaCL, pH 7.5) for 5 minutes each, and color substrate is developed using a nitroblue tetrazolium (NBT)/X-phosphate solution. The intensity of staining is then compared to the staining of a known amount of dig-labeled nucleic acid.

Northern Blot Analysis. Total cellular RNA is isolated using RNAzol (CINNA/BIOTEX) and electrophoresed on formaldehyde-denatured agarose gels as in (65). RNA is transferred onto Nytran for hybridization. Prehybridization of the filters is carried out for 4-18 hours at 37°C in 50% formamide, 5x SSC, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1 mg/ml salmon sperm DNA, 20 mM sodium phosphate (pH 6.8), and 0.1% SDS. For hybridization the appropriate 5'-end labeled oligonucleotide or ^{32}P-labelled riboprobe is added and incubated at 37° overnight. The filters are then washed in 2x SSC and 1% SDS twice at room temperature, 2x SSC and 1% SDS twice at 37°C, and 0.2x SSC and 1% SDS for 15 minutes at 37°C.

In situ hybridization. The riboprobe is degraded to 100-200 base lengths and dissolved at 3 ng/µl/kb length of cloned fragment in hybridization buffer with 20mM DTT, prior to use. Hybridization is carried out on dewaxed tissue sections at 55° overnight in 50% formamide, 0.3M NaCl, 20mM Tris-HCl, pH 8.0, 5mM EDTA, 10mM NaPO4, pH 8.0, 10% dextran sulphate, 1x Denhardts, 0.5 mg/ml yeast RNA. Slides are then washed in 50% formamide, 2x SSC, 10mM DTT at 65° for 30'; 0.5M NaCl, 10mM Tris, pH 7.5, 5mM EDTA at 37° 4 times, once including 20µg/ml RNase; and then in 2xSSC, and 0.1xSSC for 15' each at room temperature. Slides are then dehydrated and prepared for autoradiography using Kodak NTB-2 nuclear track emulsion, Kodak Developer D19, and Kodak ammonium fixer. Slides are generally developed after one week and counterstained with nuclear fast red.

Western blot: Cells or tissues are homogenized in 20mM Tris-HCl, pH 7.4, 2mM EDTA, 0.5mM EGTA for protein analysis (Bradford assay) and then electrophoresed under reducing conditions on 10% acrylamide SDS-PAGE (Laemmli) and transferred to PVDF membranes (Millipore) by the Towbin method. Membranes from each well are cut for replicate analysis, blocked in 5% non-fat dry milk, 0.5M NaCl, 10 mM Tris-HCl, pH 7.5. Primary antibodies (5-10 µg/ml) are incubated in blocking solution at RT overnight. The membranes are washed and incubated one hour at RT with biotinylated anti-

mouse/rabbit Ig (1:100, Sigma), washed, and incubated another hour with Streptavidin-alkaline phosphatase (1:200, Sigma) in 5% non-fat milk, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5. The reaction is developed with BCIP/NBT substrate. Samples are calibrated for equal loading by using antibodies against actin.

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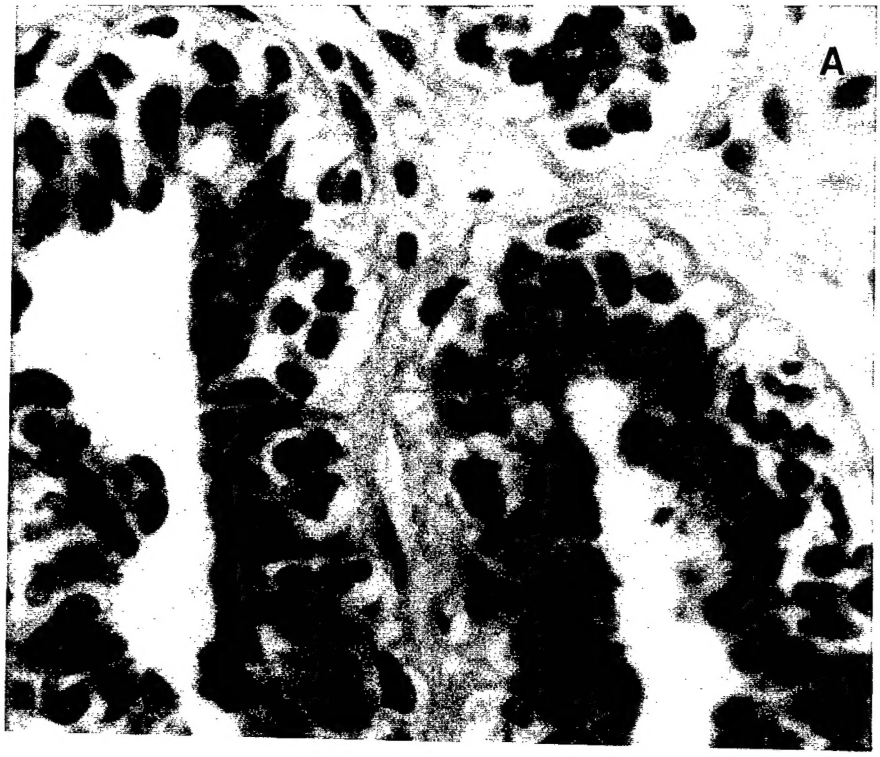
FIGURES

Figure 1. (a) immunohistochemistry showing TGFα is normally expressed in the luminal epithelial cells, (b) immunohistochemistry showing EGFR is normally expressed in the myoepithelial cells.



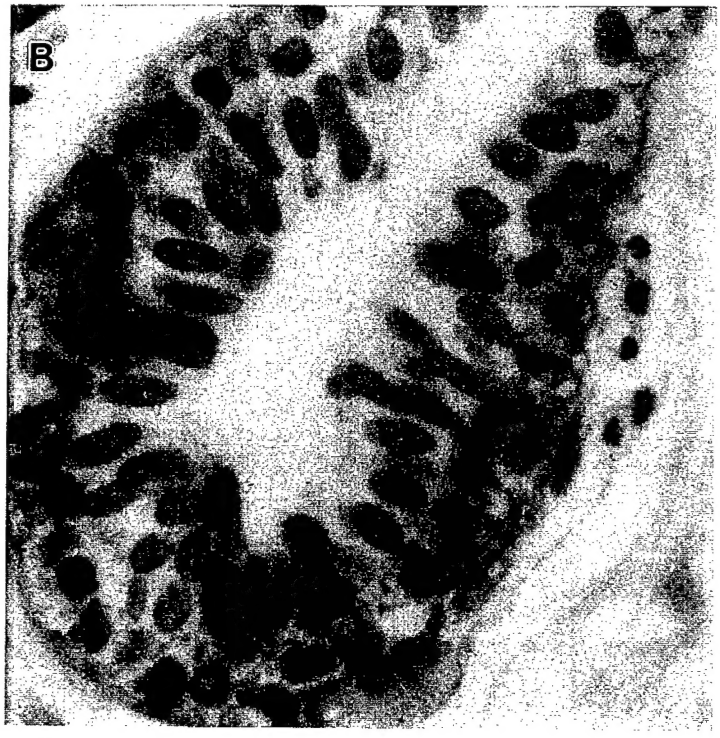


Figure 2. Northern blots (T7 is antisense probe) showing expression of TGFα mRNA using a digoxigenin-labeled riboprobe. (a) 4.8 kb and 1.6 kb TGFα mRNA is expressed in A431 cells, (b) Hs-578-Bst cells, and (c) MDA-MB-453 cells.

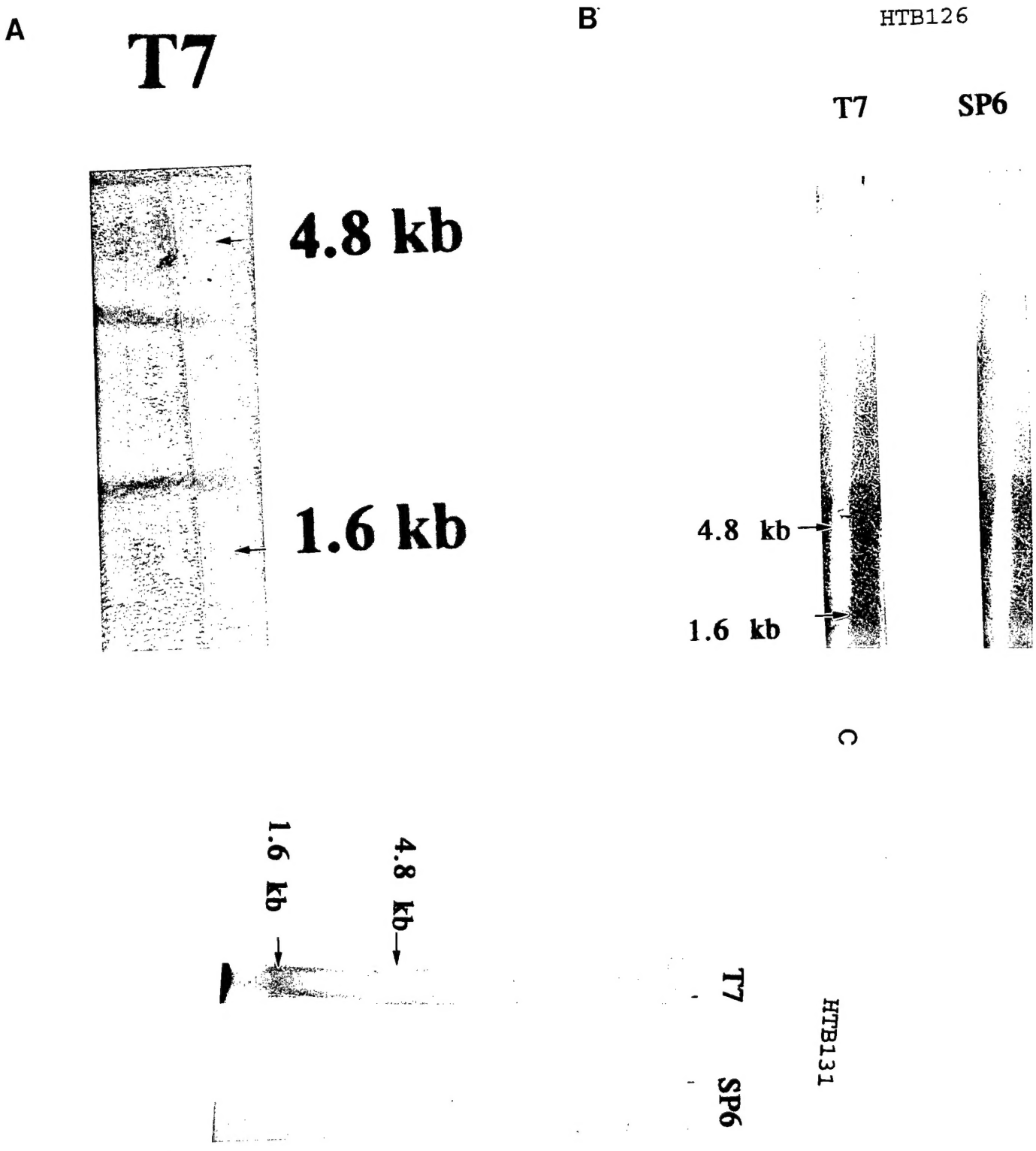


Figure 3. Western blot of protein isolated from both cell lines and fresh breast tissue samples. Lanes are protein isolated from: (a) TGFα, (b) SKBR3 cells, (c) MCF-7 cells, (d) Hs-578 Bst, (e) MDA-MB 453, (f) MDA-MB-468, (g) T-47 D, (h) cultured fibroblasts, (i) MCF-10A, (j) freshly isolated breast organoids, and (k) TGFα.

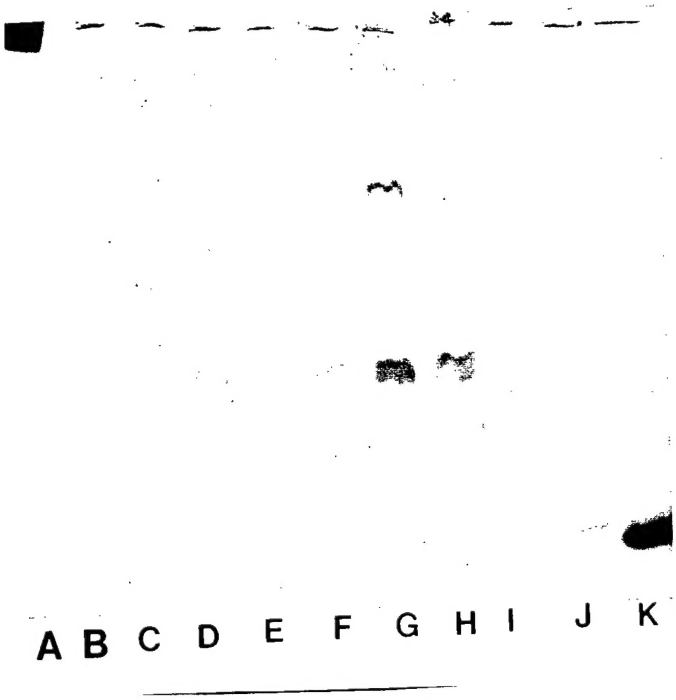


Figure 4. In situ hybridization with digoxigenin-labeled TGFα riboprobe in A431 cells. (a) antisense probe hybridizing to TGFα mRNA, (b) sense probe.